JOURNAL OF BACTERIOLOGY, Oct. 1984, p. 379–384
0021-9193/84/100379-06$02.00/0

Vol. 160, No. 1

Identification and Regulation of the *glnL* Operator-Promoter of the Complex *glnALG* Operon of *Escherichia coli*

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Received 16 March 1984/Accepted 2 July 1984

We sequenced the 274-nucleotide intercistronic *glnA-glnL* region of *Escherichia coli* to localize regulatory regions postulated from genetic evidence. The transcriptional start of *glnLp*, identified by S1 nuclease mapping, preceded the structural gene by 32 bases. NR_I, the *glnG* gene product and a repressor of *glnLp*, protected from DNase digestion a region of DNA between −12 and +15 from the transcriptional start. A mutation rendering *glnLp* insensitive to NR_I was within the protected region in a-TGCA- sequence found in all nitrogen-regulated operons, providing evidence for involvement of this sequence in interactions with NR_I. We also observed in the intercistronic region a potential *rho*-independent terminator preceding *glnLp* and a sequence previously found in other intercistronic regions.

We have previously shown that the complex *glnALG* operon of *Escherichia coli* has two operator-promoter regions (17). One is located at the beginning of *glnA*, the structural gene for glutamine synthetase, and the other is located between *glnA* and *glnL*. Consequently, transcription of the genes specifying regulatory proteins for this operon, *glnL* (*ntrB*) and *glnG* (*ntrC*) can be initiated either at *glnAp* or the weaker *glnLp* (compare data in references 17 and 22). In enteric bacteria, the product of *glnG* (*ntrC*), NR_I (nitrogen regulator I), together with the product of the unlinked *glnF* (*ntrA*) gene, is required for activation of transcription of *glnAp* in response to ammonia starvation (4, 6, 12). Furthermore, NR_I represses transcription initiated at *glnLp* (17, 20, 25). Thus, NR_I, which is also essential for the synthesis of enzymes providing the cell with ammonia and glutamate (Ntr systems), stimulates its own synthesis by activating transcription at *glnAp* in response to ammonia starvation. In cells grown with an excess of ammonia, NR_I-repressible initiation of transcription from *glnLp* provides enough NR_I to allow activation of transcription at *glnAp* upon removal of ammonia, but does not provide enough NR_I to activate the Ntr systems (13, 18). In this paper we report the nucleotide sequence of the intercistronic *glnA-glnL* region of *E. coli* containing *glnLp*, and we identify regulatory sites located in this region.

MATERIALS AND METHODS

All strains are derivatives of YMC10 (*thi endA hsr hutC*$_{Klebs}$) (2). YMC11 is like YMC10, but Δ(*glnG-glnA*)*2000*; YMC12 is like YMC10, but *glnG10*::Tn*5*; and YMC15 is like YMC10, but *glnL302*. Media and cell growth were as described previously (19).

The RNA was prepared from whole cells and hybridized to either double- or single-stranded probes as described previously (1, 3). DNA was sequenced by the methods of Maxam and Gilbert (14) and of Sanger et al. (23), as indicated below.

Hydroxylamine mutagenesis of DNA (11), "footprinting" (5), the purification of NR_I (20) and β-galactosidase assays (15) have been described previously.

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RESULTS

The region sequenced contains the carboxy terminus of the *glnA* product and the N terminus of the *glnL* product. We located the position of the carboxy terminus of glutamine synthetase, the product of *glnA*, by using plasmids carrying fragments of DNA extending from the *Cla*I site about 1,000 bases upstream from *glnA* (Fig. 1a and b) to different endpoints near the postulated carboxy terminus. We determined which plasmids restore a functional *glnA* gene, required for the ability to grow in the absence of glutamine, to strain YMC11, whose *glnALG* region is deleted. We found that pgln106, carrying DNA extending to the *Stu*I site, complemented strain YMC11, whereas pgln104, carrying DNA shorter by 135 bases and extending to the *Nru*I site, did not. Consequently, the *glnA* region determining the carboxy terminus of glutamine synthetase must be located between positions 4,570 and 4,435 on Fig. 1a. Similarly, the normal *glnL* gene carried on pgln62 restores normal regulation of glutamine synthetase formation to strain YMC15, a mutant in *glnL*; a plasmid, pgln76, constructed by fusing the *lac* promoter into the *Cla*I site at position 4,150 failed to complement YMC15 (K. Backman, personal communication). We chose therefore to sequence a stretch of DNA extending from the *Cla*I site at position 4,150 to the *Nru*I site at position 4,570, which contains the amino terminus of *glnL* and the carboxy terminus of *glnA*.

The sequence of 420 bases is shown in Fig. 2. We found that the first 42 nucleotides determined a sequence of 14 amino acids, the last 7 of which correspond to those identified as constituting the carboxy terminus of glutamine synthetase, followed by a sequence transcribed as the terminator triplet, UAA (7). The *Cla*I site located at the end of the sequence shown in Fig. 2, or at most 2 or 3 base pairs beyond, has been shown by complementation analysis to be in *glnL*, as stated in the preceding paragraph. The only open reading frame preceding this *Cla*I site starts 287 base pairs from the end of *glnA*. We assume therefore that the amino terminus of the *glnL* product is specified by the ATG triplet at this site. The putative amino acid sequence of this portion of the *glnL* product is shown in Fig. 2.

Transcriptional starts at *glnLp*. Transcription of a 270-base-pair fragment from pgln92 (Fig. 1c and d) by purified RNA polymerase had revealed a major transcriptional start at approximately 130 base pairs from the end of the fragment

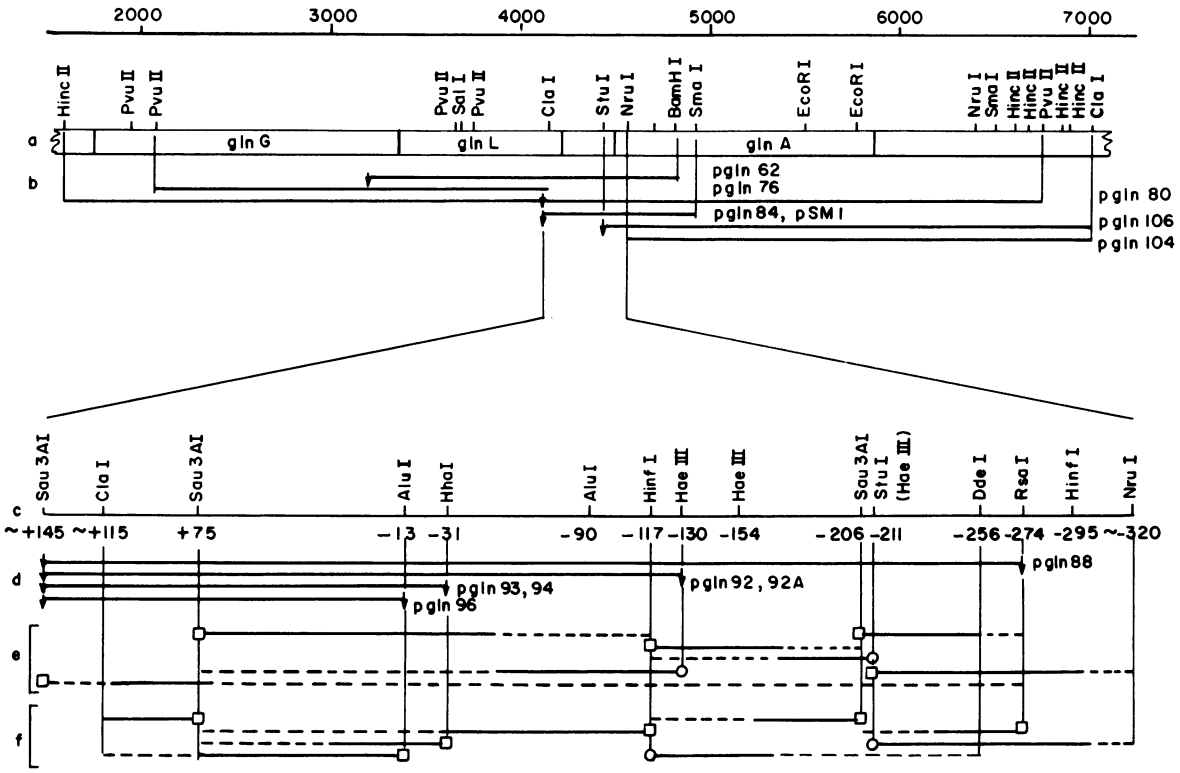

FIG. 1. Restriction map of the *glnALG* operon, plasmids used, and sequencing strategy. (a) Restriction map and genetic map of the *glnALG* operon. The coordinates were defined previously (2). (b and d) Regions cloned into pBR322. Most of the chromosomal DNA had *Hin*dIII linkers added to allow cloning into the *Hin*dIII site of pKB358. (c) Restriction map of the *glnA-glnL* intercistronic region. The coordinates are defined from the major transcriptional initiation site (see the text) and the 5′ end of the restriction sites. (e and f) Sequencing strategy for the *glnA-glnL* intercistronic region. The fragments were prepared and labeled as described previously (14). The regions sequenced are shown by solid lines. Symbols: ○, 3′ end labeled; □, 5′ end labeled. (e) Coding strand. (f) Sense strand.

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                                                                    -250
   Met-Thr-Pro-His-Pro-Val-Glu-Phe-Glu-Leu-Tyr-Tyr-Ser-Val-term     | term
5' ATG ACT CCG CAT CCG GTA GAG TTT GAG CTG TAC TAC AGC GTC TAA GTG TTT TAG TTGCCGTGGAAACTTTTCGCCTGTCTCT

3' TAC TGA GGC GTA GGC CAT CTC AAA CTC GAC ATG ATG TCG CAG ATT CAC AAA ATC AACGGCACCTTTGAAAAGCGGACAGAGA

                -200                                                -150
                 |                                                   |
GGCAGGCCTGGGATCGGTGGCAAGCACATCACGCCGGATGCGACGCAAATGCGTCTTATCCGGCCTACACGGTGATGATGTGGTAGGCCGGAGCAGGTGA

CCGTCCGGACCCTAGCCACCGTTCGTGTAGTGCGGCCTACGCTGCGTTTACGGCAGAATAGGCCGGATGTGCCACTACTACACCATCCGGCCTCGTCCACT

                -100                                                -50
                 |                                                   |
GTCGCTCTCCAACGTGAAGTTTGTCAGCTATCTGTAGCCCATCTCTGCATGGGCTTTTTTCTCCGTCAATTCTCTGATGCTTCGCGCTTTTTATCCGTAA

CAGCGAGAGGTTGCACTTCAAACAGTCGATAGACATCGGGGTAGAGACGTACCCGAAAAAAGAGGCAGTTAAGAGACTACGAAGCGCGAAAAATAGGCATT

                +1
                 |                  Met-Ala-Thr-Gly-Thr-Gln-Pro-Asp-Ala-Gly-Gln-Ile-Leu
AAAGCTATAATGCACTAAAATGGTGCAACCTGTTCAGGAGACTGCTTT ATG GCA ACA GGC ACG CAG CCC GAT GCT GGG CAG ATC CTC

TTTCGATATTACGTGATTTTACCACGTTGGACAAGTCCTCTGACGAAA TAC CGT TGT CCG TGC GTC GGG CTA CGA CCC GTC TAG GAG

Asn-Ser-Leu-Ile-Asn-Ser-Ile-Leu-Leu

AAC TCG CTG ATT AAC AGT ATT TTG TTA AT  3'

TTG AGC GAC TAA TTG TCA TAA AAC AAT TA  5'
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FIG. 2. Nucleotide sequence of the *glnA-glnL* intercistronic region. See the legend to Fig. 1 for the sequencing strategy.

and a second minor start 10 base pairs downstream. Transcription initiated at the major start was inhibited by purified NR_I, the product of the *glnG* gene (20).

We located the exact start of transcription by the S1 nuclease method (3), with RNA produced from in vitro transcription of a 270-base fragment in pgln92 (Fig. 3). The major transcriptional start is located at the first of a run of four adenine nucleotides, indicated in Fig. 2 by the superscript +1.

Similar experiments with the RNA extracted from cells confirm this position as the major transcriptional start site. Transcription starts at the same site in a strain with a mutation in *glnG*, resulting in the loss of NR_I (Fig. 4, lanes a and b) and in the wild-type strain grown in L broth-glutamine when the level of NR_I in very low (Fig. 4, lane c; Fig. 5, lane c). The *glnG* mutant cells grown on L broth-glutamine (Fig. 4, lane a) contained, in addition, some RNA capable of protecting the entire probe. The synthesis of this RNA was presumably initiated at the *glnA* promoter. We have found that this promoter can be activated weakly in the absence of the *glnG* product NR_I by the catabolite-activating protein and cyclic AMP (unpublished observation). There was very little transcription initiated at *glnLp* when the wild-type strain was grown in media with higher levels of NR_I (glucose as the major carbon source and ammonia or glutamine as the source of nitrogen) (Fig. 4, lanes d, e, and f; Fig. 5, lane b). In these cells the level of NR_I is sufficiently high to initiate transcription at the *glnA* promoter, as indicated by the fact that the entire fragment was protected from S1 nuclease hydrolysis.

In the cells grown on glucose-glutamine we observed a minor transcript with a 5′ end 15 base pairs downstream from the major start at *glnLp* (Fig. 4, lane f; Fig. 5, lane b). Previous experiments have shown that the contribution to the transcription of *glnG* from sites other than the *glnA* promoter in cells growing on glucose-glutamine is at most 2% (19). We therefore assume that this minor transcript is a physiologically unimportant degradation product of RNA whose synthesis was initiated at the *glnA* promoter, and which is only present in sufficient amount to be detected in cells grown on Ggln, when the level of RNA arising from the *glnA* promoter is highest. It appears unlikely that the initiation of transcription at a site downstream from the *glnA* promoter should be maximal when potential interference by initiation at the *glnA* promoter is greatest.

The results illustrated in Fig. 5 also permit us to compare the effect of NR_I derived from transcription of a single chromosomal *glnG* gene on either a single copy of *glnLp* in strain YMC10 (lane b) or on the multiple copies of *glnLp* present in strain YMC10(pgln84) (lane d), keeping in mind that a 10-fold higher concentration of RNA was used in the case of strain YMC10. At this intracellular concentration, NR_I can almost completely prevent the initiation of transcription of a single *glnLp* promoter, but not of the multiple promoters present in the plasmid-carrying strain. The transcriptional starts are the same whether *glnLp* is carried on the chromosome or the plasmid.

We had previously shown that strains carrying plasmid pgln94, which has the same fusion of *lacZ* to *glnL* as plasmid pgln84 (Fig. 1d), but whose bacterial DNA only extends to an *Hha*I site, have a β-galactosidase level approximately 10 times higher than that found in strains carrying pgln84 (25).

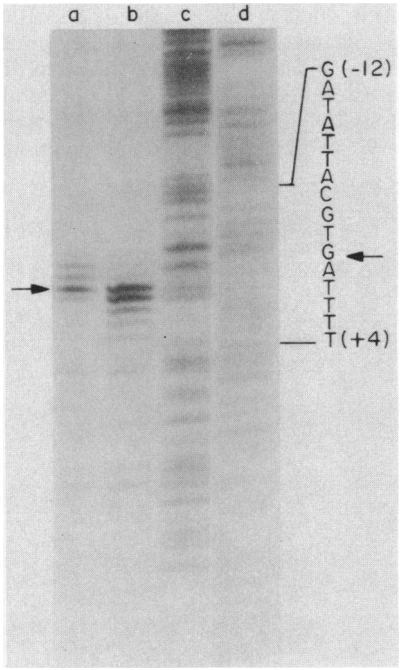

FIG. 3. Locating the transcriptional start of *glnL* mRNA synthesized in vitro. The mRNA was made as described previously (20) and hybridized to an asymmetrically 5′ end-labeled, double-stranded fragment from positions +75 (*Sau*3AI site) to −130 (*Hae*III-*Hin*dIII linker) (Fig. 2) and digested with 27 U (lane a) or 130 U (lane b) of S1 nuclease at 37°C for 10 min after adding 10 μg of tRNA. The products were subjected to electrophoresis in a 15% gel next to the A+G (lane c) and C+T (lane d) sequencing reactions.

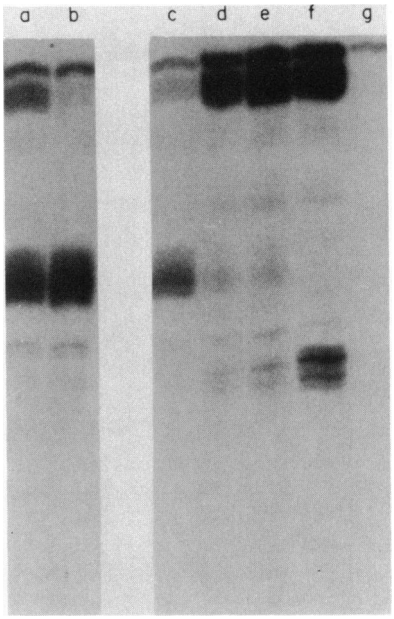

FIG. 4. Transcriptional starts from *glnLp*. A 200-μg sample of RNA was hybridized to a single-stranded 5′ end-labeled probe from positions +115 (*Cla*I site) to −31 (*Hha*I site) (Fig. 2). The hybrids were digested with 80 U of S1 nuclease for 40 min at 35°C. The RNA samples were from the following sources: a *glnG* strain (YMC12) grown in L broth-glutamine (lane a) or 0.4% glucose–0.2% $(NH_4)SO_4$–0.2% glutamine (lane b); a wild-type strain (YMC10) grown in L broth–0.2% glutamine (lane c), 0.4% glucose–0.2% $(NH_4)_2SO_4$–0.2% glutamine (lane d), 0.4% glucose–0.2%–$(NH_4)_2SO_4$ (lane e), and 0.4% glucose–0.2% glutamine (lane f); and yeast tRNA (lane g).

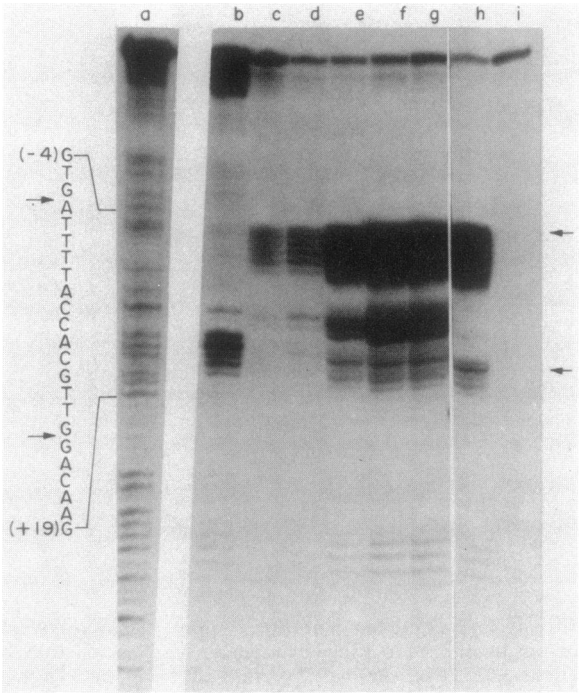

FIG. 5. Transcriptional starts from plasmid *glnLp*. The procedure, probe, and abbreviations are the same as used in Fig. 4. Lane a is the A+G sequencing reaction. In lanes b and c, 200 μg of RNA was from wild-type *E. coli* (YMC10) without plasmid grown in 0.4% glucose–0.2% glutamine (lane b) or L broth–0.2% glutamine (lane c). In lanes d through g, 20 μg of RNA was from wild-type *E. coli* with either a plasmid with a wild-type *glnLp* (YMC10[pgln84]) grown in 0.4% glucose–0.2% glutamine (lane d) or 0.4% glucose–0.2% $(NH_4)_2SO_4$–0.2% glutamine (lane e) or a plasmid with a mutant *glnLp* (YMC10[pSM1]) grown in 0.4% glucose–0.2% glutamine (lane f) or 0.4% glucose–0.2% $(NH_4)_2SO_4$–0.2% glutamine (lane g). In lane h, 2 μg of RNA was from wild-type *E. coli* bearing a plasmid with a strong *glnLp* (YMC10[pgln94]) grown in 0.4% glucose–0.2% $(NH_4)_2SO_4$–0.2% glutamine. Lane i contained 200 μg of yeast tRNA; in each case, carrier tRNA was added to bring the total RNA to 200 μg.

Transcription starts at the same site in both plasmids, but results in the production of a considerably greater amount of *glnL*-specific RNA in the case of pgln94: 2 μg of RNA isolated from strain YMC10(pgln94) is as effective in protection of the DNA from digestion by S1 nuclease as 20 μg of RNA isolated from strain YMC10(pgln84) (Fig. 5, lanes e and h).

Mutations rendering transcription initiated at *glnLp* insensitive to NR_I. We have previously shown that NR_I, the product of *glnG*, represses β-galactosidase in strains carrying plasmid pgln84, which contains the intercistronic *glnA-glnL* region as well as the amino terminus of *glnL* fused in phase to *lacZ* lacking a translational start. Consequently, colonies of strain YMC15 whose level of the *glnG* product is always elevated are only faintly blue when grown on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside, an indicator for β-galactosidase. We used plasmid DNA that had been treated with hydroxylamine to transform strain YMC15, and we selected dark blue colonies on indicator plates. The synthesis of β-galactosidase driven from the wild-type *glnLp* of pgln84 was repressed 10-fold by the *glnG* product, but only 2.6-fold in a mutated plasmid (pSM1) (data not shown). We used fragments produced by the digestion of pgln84 and pSM1 with *Sau*3AI endonuclease (Fig. 1c) as templates for transcription by purified RNA polymerase to test the effect of NR_I protein. A fivefold excess of NR_I over DNA almost completely prevented the initiation of transcription at the major transcriptional start site of the normal *glnLp*, but had little effect on the mutant *glnLp* (Fig. 6). The minor transcriptional start site yielding an RNA shorter by 10 nucleotides was not greatly affected by the presence of NR_I in either case.

The site of initiation of transcription from cells carrying *glnLp*-containing plasmids was also determined by S1 nuclease mapping. In cells carrying pgln84 wild-type *glnLp* there is considerably less initiation of transcription at *glnLp* during growth on glucose-glutamine, when the level of NR_I is high, than on glucose-ammonia-glutamine, when the level of NR_I is lower (Fig. 5, lanes d and e). On the other hand, in cells carrying pSM1 there is strong initiation of transcription at this promoter irrespective of the level of NR_I (Fig. 5, lanes f and g).

Sequencing of the *Sau*3AI fragment (position +75 to −206) of the plasmid pSM1 after cloning into M13mp8 with the dideoxy method (23) revealed that the mutation had resulted in the substitution of a thymine for cytosine in position −4 of the sequence shown in Fig. 2, indicating that NR_I binds in close proximity to the initiation site.

NR_I protein protects a portion of DNA from the *glnLp* region against digestion by DNase. We used the *Hin*dIII (−31)–*Sau*3AI (+75) fragment of pgln94, labeled at the *Hin*dIII end of the sense strand, and an *Hha*I (−31)–*Cla*I (~ +115) fragment of pgln92 labeled at the *Cla*I end of the coding strand to determine the sites of binding of NR_I to the *glnLp* region. The results show on the noncoding strand protection of a 5′AATGCA3′ fragment separated by three nonprotected bases, CTA, from another protected 5′AAATG3′ fragment (Fig. 7). This region extends from positions −8 to +6 on the sequence shown in Fig. 2. Another weakly protected region, 5′AACCT3′, extends from positions +11 to +15 (Fig. 7). On the coding strand 5′ATAG3′ (positions −9 to −12) and 5′GCAC3′ (positions +10 to +7)

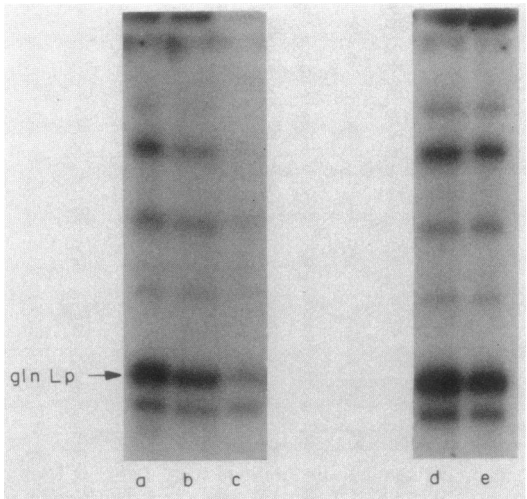

FIG. 6. Effect of NR_I on transcription in vitro from *glnLp*. The DNA template was either from pgln84 (wild-type *glnLp*) (lanes a through c) or from pSM1 (mutant *glnLp*) (lanes d and e). The ratio of NR_I to DNA in lanes a through e was no NR_I, 2, 5, no NR_I, and 5, respectively (see reference 20 for interpretation of the other bands and details of the assay).

are strongly protected, between them, weak protection is extended to 5'TA3' (positions −1 to −2) (data not shown). When these sites are displayed on a three-dimensional model, all of the protected bases appear on the same side of the DNA helix.

DISCUSSION

We had previously shown that the transcription of the *glnLG* segment of the complex *glnALG* operon can be initiated at a site in the intercistronic *glnA-glnL* region and that it is subject to repression by NR_I, the product of *glnG* (17, 20, 25). Our present results confirm these conclusions and reveal the structure of DNA at the sites responsible for the initiation of this transcription and interaction with NR_I.

The determination of the DNA sequence of the intercistronic region has shown the probable start of translation of the *glnL*-specific mRNA. At the appropriate distance, 8 nucleotides from the initiating AUG triplet, this RNA contains the characteristic AGGAG sequence necessary for the interaction with 16S RNA (24).

The transcriptional start is located 32 base pairs upstream from the translational start. It is preceded by the canonical -TATAAT- sequence in positions −11 to −6 (Fig. 2) (9). Still further upstream we find between −36 and −26 the sequence -TTCGCGCTTT-, which resembles the canonical "−35" sequence, -TTGACA.TT-. In plasmid pgln94 (Fig. 1), an *Hin*dIII linker has been positioned at an *Hha*I cleavage site at position −31 (Fig. 2). This fusion alters the sequence between positions −33 and −25 to -TTGCTTTTT-, a sequence with the TTG triplet of the consensus "−35" sequence, which is lacking in the original sequence. This alteration is apparently responsible for the 10-fold increase in the expression of the *glnL* gene previously reported (25). Conversely, fusing the *Hin*dIII linker at position −12 results in the loss of a sequence resembling the canonical "−35" sequence and in diminished ability to express the *glnL* gene.

The NR_I protein binds to a stretch of DNA extending on one face of the double helix from positions −12 to +15 (Fig. 2). Our estimate of the size of NR_I, a dimer consisting of two identical subunits with molecular weights of 55,000 (20), is compatible with the binding of one molecule of NR_I to this site.

Our finding that a mutation changing the C located at position −4 to a T greatly reduces the ability of NR_I to block the initiation of transcription emphasizes the importance of the -AATGCA- sequence extending from positions −8 to −3 in the interaction of the DNA with NR_I. It is of particular interest that Ow et al. (16) discovered the presence of the sequence -TGCA- in the promoter regions of all four genes of *E. coli* (*glnA*), *Klebsiella pneumoniae* (*nifL*), and *Salmonella typhimurium* (*dhuA* and *argT*) that have been examined whose expression requires activation by the products of *glnG* and *glnF*. In the case of the *glnL* promoter-operator, this sequence appears twice in the segment of DNA to which NR_I, the product of *glnG*, was found to bind at positions −6 to −3 and at positions +8 to +11. The fact that NR_I represses transcription originating at the *glnL* promoter, but appears to activate transcription initiated at the *glnA*, *nifL*, *dhu*A, and *argT* promoters, suggests that the distance of the NR_I binding site from the transcriptional start site may determine whether activation of transcription or repression results from the interaction. The fact that in the case of *glnL* the binding site overlaps both the "−10" region and the transcriptional start site explains the effectiveness of NR_I as a repressor of the *glnLG* operon.

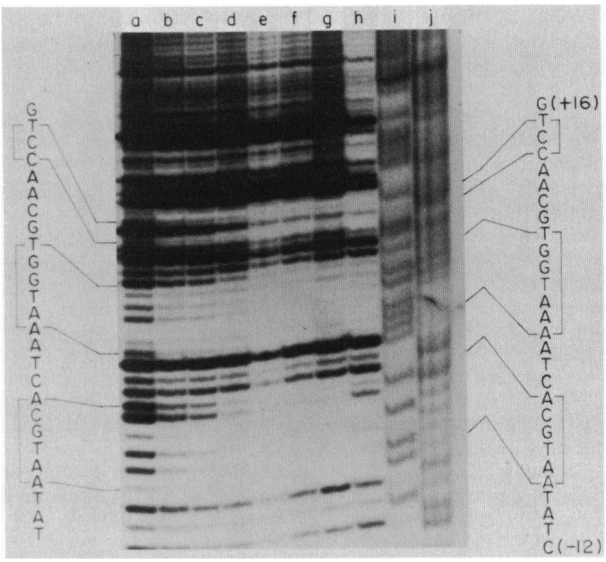

FIG. 7. NR_I protection of *glnLp* from DNase I digestion. Approximately 0.5 pmol of a fragment from positions +75 to −31 labeled at the 5' end of the upstream cut was mixed with 0, 0.5, 1, 2, 4, 6, 12, and 12 pmol of NR_I (lanes a through h, respectively) at room temperature for 15 min. Then DNase was added, and the reaction proceeded for 3 min (lanes a through g) or 10 min (lane h) and was terminated with EDTA. The DNA was ethanol precipitated and electrophoresed into a 15% gel next to the chemical sequencing reactions A+G (lane i) and C+T (lane j).

Another feature of the sequence in the intercistronic *glnA-glnL* region may explain the observation that the *glnG* gene is less frequently transcribed than the *glnA* gene when transcription of both genes is initiated at the *glnA* promoter (17). The RNA resulting from transcription of the DNA between positions −79 and −57 can give rise to a G+C-rich stem-loop structure followed by six uridine residues. This structure has the characteristic features of an *rho*-independent terminator and may cause termination of a portion of the transcripts initiated at *glnAp* (21).

Also deserving comment is the structure of the RNA that would result from transcription of DNA between positions −188 and −100. The sequence of the RNA, which may assume a stem-loop structure, has great similarity to a sequence resulting from the transcription of many intercistronic regions, such as the one in the region between *lamB* and *molA* of *E. coli* (10).

It is of interest that a large portion of the nucleotide sequence of the intercistronic *glnA-glnL* region of *S. typhimurium* is almost identical with that of *E. coli* (Fig. 2; underlining indicates identity in the DNA of the two organisms). In particular, the sequences coding for the C-terminal and N-terminal portions of the products of the *glnA* and *glnL* genes, respectively, the sequence between positions −36 and +33 determining the initiations of transcription and translation and the interaction with NR_I, and the sequence presumably responsible for the partial termination of transcription initiated at *glnAp* between positions −79 and −57 are almost identical. On the other hand, there is almost no similarity in the nucleotide sequences of the DNA from the two organisms between positions −220 and −82 (8). So far, no major differences in the regulation of expression of the complex *glnALG* operons of *E. coli* and *S. typhimurium* have been recorded. It would therefore appear that the ability of the RNA originating from transcription of the DNA

between positions −188 and −100 to form complex stem-loop structures in *E. coli* does not play an important part in the regulation of the expression of the *glnALG* operon.

ACKNOWLEDGMENTS

We acknowledge the valuable advice of Rex Chisholm, Masataka Nakamura, and Teresa A. Webster concerning the DNA sequencing. We also acknowledge the help of Jin-Soon Moon in some of the experiments.

This work was supported by Public Health Service research grants GM-07446 and AM-13894 from the National Institute of General Medical Sciences and the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, respectively, and by grant PCM78-08576 from the National Science Foundation.

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